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Impaired DNase1-mediated degradation of neutrophil extracellular traps is associated with acute thrombotic microangiopathies

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Abstract Main Body

Running title: Impaired NET-degradation in TMAs

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Summary

Background - Acute thrombotic microangiopathies (TMAs) are characterized by excessive microvascular thrombosis and are associated with markers of neutrophil extracellular traps (NETs) in plasma. NETs are composed of DNA fibers and promote thrombus formation through the activation of platelets and clotting factors.

Objective - The efficient removal of NETs may be required to prevent excessive thrombosis such as in TMAs. To test this hypothesis, we investigated whether TMAs are associated with a defect in the degradation of NETs.

Approach and Results - We show that NETs generated *in vitro* were efficiently degraded by plasma from healthy donors. However, NETs remained stable after exposure to plasma from TMA patients. The inability to degrade NETs was linked to a reduced DNase activity in TMA plasma. Plasma DNase1 was required for efficient NET-degradation and TMA plasma showed decreased levels of this enzyme. Supplementation of TMA plasma with recombinant human DNase1 restored NET-degradation activity.

Conclusions - Our data indicates that DNase1-mediated degradation of NETs is impaired in patients with TMAs. The role of plasma DNases in thrombosis is, as of yet, poorly understood. Reduced plasma DNase1 activity may cause the persistence of pro-thrombotic NETs and thus promote microvascular thrombosis in TMA patients.

Key words: Neutrophils, Neutrophil Extracellular Traps, Deoxyribonuclease I, Thrombosis, Thrombotic Microangiopathies

Introduction

Thrombosis is a major cause of morbidity and mortality. Current antithrombotic therapies predominantly inhibit platelet aggregation or fibrin formation. We, along with others, have recently discovered that neutrophil extracellular traps (NETs) are a stimulus for thrombus formation [1, 2], thereby offering a new diagnostic and therapeutic target for thrombotic diseases.

NETs are composed of extracellular DNA fibers, which are associated with histones and neutrophil enzymes [3]. NETs are released by activated neutrophils during thrombus

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formation [4]. The formation of NETs involves the unwinding of compact DNA fibers of the nucleus, followed by the breakdown of the nuclear envelope [5, 6]. In the final stage of NET formation, NETs are discharged by an active mechanism or after cytolysis [5, 7]. NET formation is mediated by peptidylarginine deiminase 4 (PAD4) and neutrophil elastase (NE), which modify histones and enable DNA decondensation [6, 8]. Neutrophils from mice, which are deficient in PAD4 or NE, cannot form NETs [6, 8]. NETs promote thrombosis by binding and activating platelets as well as clotting factors. Mechanistically, histones in NETs activate platelets and exacerbate platelet aggregation [1, 9, 10]. NET-DNA serves as a negatively charged surface, similar to platelet polyphosphates [11] or extracellular nucleic acids [12], to initiate Factor XII activation [4]. Additionally, the tissue factor pathway is activated by neutrophil serine proteases in NETs, by cleaving tissue factor pathway inhibitor [2, 4]. NETs are abundant in thrombi from animals and patients [1, 4, 13, 14] and inhibiting NET formation prevents experimental thrombosis [6, 8], illustrating the importance of NETs for thrombus formation.

We and others have previously identified markers of NETs in plasma from patients with thrombotic diseases including TMAs [15], deep vein thrombosis and atherosclerosis [16-18]. The highest levels of NET-markers, namely DNA/histone complexes, myeloperoxidase and S100A8/A9, were observed in plasma from patients with acute TMAs [15]. TMAs are a heterogeneous group of life-threatening conditions characterized by disseminated microvascular thrombosis with thrombocytopenia, fragmentation of erythrocytes and ischemic organ damage [19, 20, 21]. TMAs include thrombotic thrombocytopenic purpura (TTP), a subtype of TMAs often associated with severe deficiency in ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13), and typical hemolytic uremic syndrome (HUS), a TMA subtype often precipitated by an infection with enterohemorrhagic *E. coli* producing shigatoxin. TMAs are associated with additional conditions including hereditary and acquired hyper-activatability of the alternative complement pathway (atypical HUS), disseminated neoplasia, certain drugs, pregnancy and autoimmune diseases illustrating the heterogeneity of TMA pathophysiology [19, 20, 21]. We identified markers of NETs in TMAs of various pathophysiological causes including TTP, enteropathogenic *E. coli*-associated HUS and tumor-associated TMA [15].

We hypothesized that timely and efficient removal of NETs is required to prevent excessive thrombus formation. As a first step towards addressing this hypothesis, we investigated whether disseminated microvascular thrombosis in acute TMAs is associated with a defect in the degradation of NETs. It is known that NETs are not stable when incubated in serum from animals and humans [22, 23]. We therefore analysed the stability of NETs exposed to plasma from TMA patients and healthy donors.

Materials and Methods

Blood collection

Peripheral blood samples for neutrophil isolation or plasma preparation were collected from healthy volunteer donors recruited by the Institute of Transfusion Medicine of the University Medical Center Hamburg-Eppendorf. For this study only residual amounts of peripheral blood samples have been used, which had been taken routinely from healthy blood donors. Blood donors gave general written informed consent to the use of their blood samples in scientific studies, which would have been discarded otherwise. All samples were anonymized before inclusion in this study.

Neutrophil isolation

Peripheral blood was anticoagulated with ethylenediaminetetraacetic acid (K2 EDTA monovette, Sarstedt) and neutrophils were isolated as previously described [5]. In brief, blood was layered onto Histopaque 1119 (Sigma Aldrich). After centrifugation for 20 min at 800 g, the neutrophil-rich layer was collected. The cells were washed with Hanks-buffered salt solution without divalent cations (HBSS-, Life Technologies) supplemented with 5 mM EDTA and 0.1% bovine serum albumin (BSA, Sigma-Aldrich). Washed cells were further fractionated on a discontinuous Percoll gradient (GE Healthcare). After centrifugation for 20 min at 800 g, the neutrophil-rich layer was collected and washed with 0.1% BSA in HBSS-. All procedures were conducted at room temperature. Neutrophil viability was greater than 98%, as determined by trypan blue (Sigma-Aldrich) exclusion.

Plasma preparation

Plasma was prepared by centrifugation of citrated blood (Coagulation monovette, Sarstedt) for 10 min at 3000 g. Supernatant plasma was collected and re-centrifuged for 3 min at 10.000 g. Double-centrifuged platelet-poor plasma was stored in aliquots at -70°C.

TMA patient plasma samples

We used a previously described collection of plasma samples from patients with acute TMA [15]. Plasma samples were selected from patients referred for ADAMTS13 activity testing for diagnostic purposes to the Hemostasis Research Laboratory, Department of Hematology, Bern University Hospital and the University of Bern (Bern, Switzerland). All patients had received a diagnosis of TMA by their referring physicians defined by microangiopathic hemolytic anemia with schistocytes on the blood smear and thrombocytopenia with or without clinically apparent ischemic organ dysfunction. In patients treated by plasma exchange therapy and fresh frozen plasma replacement, plasma was collected before initiation of plasma therapy. The plasma collection also included plasma from 10 healthy donors, which served as control samples for TMA patient plasma analysis. All samples were stored in aliquots at -70°C. The study was approved by the responsible Ethics Committee (Kantonale Ethikkommission, Bern, Switzerland).

Commercial DNase1

We used recombinant human DNase1 (rhDNase1, dornase alpha, Pulmozyme; Roche) as commercial source of DNase1. Dilutions of DNase1 were made in phosphate-buffered saline (PBS) containing 0.1 % BSA.

Actin:G1 complex

Rabbit skeletal muscle actin was prepared from dried acetone powder obtained from fresh rabbit psoas muscle as previously described [24]. G-actin was stored in G-buffer containing 5 mM HEPES pH 7.4, 0.1 mM CaCl₂, 0.5 mM NaN₃, and 0.2 mM ATP. The N-terminal segment 1 (G1) of gelsolin was recombinantly expressed in *Escherichia coli* and purified from supernatants of bacterial homogenates [25]. In order to generate the actin:G1 complex, both proteins were mixed at equimolar concentration in G-buffer [26].

NET-degradation assay

NET-degradation was analyzed according to the protocol of Hakkim et al. [22] with modifications. Purified neutrophils in serum-free Dulbecco's Modified Eagle's medium (DMEM, Life Technologies) were seeded to sterile 96 well plates (Falcon) at a concentration of 5×10^4 cells per well. To induce NET-formation, neutrophils were activated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 4 hours at 37°C with 5% CO₂ and humidity [5]. NETs generated under these conditions are stable for up to 7 days [22]. We stored 96 well plates with NETs at 4°C for up to 24 hours. To test the stability of NETs in plasma, we diluted citrated plasma in HBSS with divalent cations (HBSS+, Life Technologies) containing 20 µM of the thrombin inhibitor D-Phe-Pro-Arg-chloromethylketone (PPACK, Santa Cruz). PPACK was added to prevent plasma clotting while enabling calcium-dependent mechanisms. NETs were incubated with diluted plasma for 6 hours at 37°C with 5% CO₂ and humidity. NET-degradation was stopped by replacing the diluted plasma with 2% paraformaldehyde (PFA, Sigma-Aldrich) in PBS. After incubation for 1 hour at RT, the plates were washed twice with PBS. Nuclei and NETs were then labeled fluorescently by adding 2 µM of the DNA dye SytoxGreen (Life Technologies). The total fluorescence of each well was quantified using a fluorometer (Tecan Genios). Images of fluorescently stained nuclei and NETs were acquired with an inverted fluorescence microscope (Zeiss Axiovert 200M).

Single radial enzyme diffusion (SRED) assay

We quantified DNase activity by the SRED assay [27]. This involved agarose gels containing fluorescent double-stranded DNA being prepared by dissolving 0.13 mg/ml DNA from salmon testes (Sigma-Aldrich) in buffer with 100 mM MES pH 6.5, 20 mM MgCl₂, 2 mM CaCl₂, and 2x SYBR Safe (Life Technologies). The DNA solution was heated at 50°C for 10 min and mixed with an equal volume of 2% agarose GP-36 (Nacalai Tesque). The mixture was poured into trays and stored at RT until solidification. Two µl of sample were applied to wells of 1.0 mm radius. After 24 hour incubation at 37°C in a humid chamber, the fluorescence of the gels was recorded with a fluorescence scanner (Molecular Imager FX, Bio-Rad). Image J (NIH) was used for the quantification of signal intensity and the radius of the circles reflecting DNase activity.

Denaturing SDS-PAGE zymography (DPZ)

To characterize DNases we applied the DPZ method [28]. In brief, SDS-PAGE gels were prepared with 4% (v/v) stacking gels without DNA and 12% (v/v) resolving gels containing 200 µg/ml of salmon testes DNA. Two µl of plasma were mixed with 5 µl of water and 5 µl SDS gel-loading buffer (BioRad), boiled for 5 minutes, and loaded onto the gels. PageRuler Prestained Protein Ladder (MBI Fermentas) was used as molecular marker. Electrophoresis was carried out at 120 V using Tris/glycine electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.7). After electrophoresis, SDS was removed by washing the gels with 10 mM Tris/HCl pH 7.8 for 30 min at 50°C twice, and the proteins were refolded by incubating the gels overnight at 37°C in a solution containing 5% (w/v) milk powder, 10 mM Tris/HCl pH 7.8, 3 mM CaCl₂, 3 mM MgCl₂, 100 U/ml penicillin and 100 µg/ml streptomycin. Next, the gels were transferred to a buffer containing 10 mM Tris/HCl pH 7.8, 3 mM CaCl₂, 3 mM MgCl₂, 100 U/ml penicillin, 100 µg/ml streptomycin and 1x SYBR Safe. Gels were incubated for 12 hours at 37°C and fluorescence was recorded by a fluorescence scanner. Image J was used for the quantification by measuring the intensity of bands.

Statistical evaluation

Statistical analysis was performed using Prism Software (GraphPad) and included mean ± SD, linear regression analysis, paired t-test, Mann-Whitney test, Kruskal-Wallis test with Dunn's post hoc test and results were considered significant at $p < 0.05$. Spearman's rank correlation coefficients were calculated with Bonferroni-adjusted significance levels and considered significant at $p < 0.005$.

Results

Plasma from healthy donors degrades NETs efficiently

We speculated that the microvascular thrombotic process in patients with TMA is linked to a defect in the degradation of NETs. To test our hypothesis, we compared the stability of NETs after exposure to TMA patient plasma or plasma from healthy controls. We incubated purified neutrophils from healthy donors, which had been activated to release NETs, with 5% citrated plasma from healthy control donors or buffer for 6 hours. NET-degradation was measured by two approaches. In the first method, we fluorescently labeled the DNA of neutrophils and

NETs and analyzed the samples by fluorescence microscopy. In samples containing buffer, NETs were identified as elongated DNA fibers and nuclei as dots (Fig. 1A). Incubation with plasma from healthy control donors dissolved NETs, but not the nuclear staining pattern (Fig. 1B, C). A similar staining was observed using anti-DNA antibodies instead of fluorescent DNA dyes (Fig. S1). To assess NET-degradation quantitatively, we measured the fluorescence intensity after incubation with plasma. We observed a concentration dependent decrease of the DNA fluorescence (Fig. 1D). DNA fluorescence reached a minimum at plasma concentrations of 2.5% or higher. At these concentrations the DNA fluorescence is reduced by 75% (Fig. 1D). The remaining 25% DNA-fluorescence is emitted from nuclei rather than NETs (Fig. 1B, shaded area in Fig. 1D). Taken together, these results indicate that NETs are more sensitive to digestion by plasma than neutrophil nuclei and that plasma from healthy donors degrades NETs efficiently.

NET-degradation by plasma from TMA patients is impaired

Next, we explored the effect of TMA patient plasma on NETs. We analyzed citrated plasma from 27 patients with acute TMAs of various etiologies (Table S1). We tested plasma collected from 3 patients with enteropathogenic *E.coli*-associated HUS, 8 patients with acute acquired TTP, 7 patients with tumor-associated TMA, and 9 patients with TMAs of unknown etiology as well as control plasma obtained from 10 healthy donors. Neutrophils, which had been activated to release NETs, were incubated for 6 hours with 5% patient plasma, plasma from healthy control donors or buffer. NET-degradation was analyzed by microscopy and quantified by measuring the intensity of DNA fluorescence. We observed complete degradation of NETs by all 10 control plasma samples, whereas NETs were still visible after exposure to plasma from 21 of 27 TMA patients (Fig. 2A). Quantification of DNA fluorescence intensity revealed that plasma from healthy controls, but not TMA patient plasma, decreased the DNA fluorescence by 75% compared to samples incubated with buffer (Fig. 2B). These data suggest that plasma-mediated degradation of NETs is reduced in patients with acute TMAs of various etiologies.

DNase activity is decreased in TMA patient plasma

Double-stranded DNA fibers are the major structural component of NETs and make NETs sensitive to digestion by DNases [29]. DNases require calcium and magnesium cations for optimal activity and NETs were stable when incubated with plasma in the presence of EDTA, a calcium and magnesium chelator (Fig. S1). We therefore analyzed whether DNase activity

in plasma from TMA patients is reduced compared to healthy control donors. Plasma DNase activity was measured by the SRED assay [27]. In this method, plasma is loaded onto an agarose-gel, which contains fluorescently labeled double-stranded DNA. Degradation of DNA by plasma DNases, which diffuse into the agarose gel, can be quantified by a decrease in fluorescence intensity. These experimental conditions allow the quantification of very low levels of DNase activity [30]. Using a standard curve of rhDNase1, we estimated the average DNase activity in citrated plasma from healthy control donors to be 0.32 ± 0.21 mU/ml ($n = 10$, \pm SD), which is within the range of previously reported DNase activities in human serum [27]. Because our study included plasma samples stored at -70°C for several years, we questioned whether freeze-thawing or prolonged storage of plasma reduces DNase activity. We analyzed plasma collected from healthy donors. We observed similar DNase activities in fresh plasma, plasma subjected to 20 freeze-thawing cycles and plasma stored at -70°C for at least 7 years (data not shown), indicating that enzymatic activity of DNase is not compromised by our storage conditions. Analysis of plasma from patients with acute TMA showed a significant reduction in DNase activity compared to healthy controls (Fig. 3A). We observed DNA fluorescence of 50% or higher, indicating strong inhibition of NET-degradation, only in samples containing 0.12 mU/ml or less of DNase activity (Fig. 3B).

Decreased DNase activity was also associated with increased levels of DNA [15] in these samples (Table S1; DNA vs DNase1, Spearman r : -0.41, $p = 0.01$) suggesting that reduced DNase activity may lead to higher levels of NET-markers in patients with acute TMA.

NET-degradation requires plasma DNase1 activity

DNase1 and DNase γ are the predominant extracellular DNases in circulation [31]. To test which DNase is required for NET-degradation, we pre-incubated plasma with monomeric actin, which inhibits the enzymatic activity DNase1 but not DNase γ [32]. We supplemented plasma from healthy controls with the complex of monomeric actin with gelsolin segment 1 (G1). G1 keeps actin in its monomeric state, prevents actin polymerization in plasma and thus maintains the DNase1-inhibitory activity of actin [26]. Actin:G1 supplemented plasma showed no NET-degrading activity indicating that NETs are degraded by plasma DNase1 (Fig. 4A, B).

Levels of active DNase1 are decreased in TMA patient plasma

We now analyzed whether impaired NET-degradation by TMA patient plasma is due to reduced levels of DNase1 in plasma. To detect DNase1 in human plasma, we employed commercially available techniques including an ELISA and Western blotting based on a polyclonal antibody against DNase1. However, both approaches failed to detect DNase1 in plasma from control donors due to a lack of sensitivity or specificity, respectively (Fig. S2). Therefore we quantified DNase1 levels in human plasma by denaturing SDS-PAGE zymography (DPZ) method [28], instead of antibody-based techniques. The DPZ method separates SDS-denatured plasma proteins in a polyacrylamide gel, which contains DNA. Refolding of the proteins after SDS elution restores the enzymatic activity of DNases. Fluorescent staining of DNA in the gels detects DNase activity as an unlabeled band. DPZ analysis of plasma from healthy controls revealed a single protein band with DNase activity of approximately 38 kDa (DPZ in Fig. 5A). The size of this DNase is within the reported size of DNase1 in human serum [33]. Using this approach, we quantified strongly reduced levels of active DNase1 in TMA patient plasma compared to healthy controls (Fig. 5A, 5B).

DNase1 detected by the DPZ assay is, unlike the SRED method, not sensitive to inhibition by actin. The addition of actin:G1 to plasma dose-dependently blocked DNase1 activity detected by the SRED assay, but had no effect on DNase1 measured by DPZ (Fig. 5C, 5D). Interestingly, plasma DNase1 activities determined in TMA patients by the DPZ and SRED assay are strongly correlated (Fig. 5E) indicating that the reduction of DNase1 activity is not due to an inhibitor but a result of lower DNase1 levels.

RhDNase1 restores NET-degradation by TMA patient plasma

Finally, we questioned whether NET-degradation activity of TMA patient plasma can be restored by supplementing plasma with rhDNase1. We mixed TMA patient plasma with 0.5 mU/ml rhDNase1. Analysis by the SRED assay showed that the addition of rhDNase1 restored DNase activity in TMA patient plasma to levels detected in healthy control donors (Fig. 6 A, B). Analysis by NET-degradation revealed that the supplementation with rhDNase1 enables TMA patient plasma to degrade NETs efficiently (Fig. 6 C, D). We furthermore analyzed whether plasma from healthy donors could serve as a source of DNase1 and restore NET-degradation by TMA patient plasma. Indeed, supplementing plasma from TMA patients with a pool of plasma from healthy donors at a 1:1 ratio (v/v) enabled NET-degradation (Fig.

6 C, D). In summation, these data indicate that the defect in DNase1-mediated NET-degradation can be restored by rhDNase1 or plasma from healthy donors.

Discussion

Our data suggests that lowered levels of plasma DNase1 lead to an impaired NET-degradation in vitro and are associated with acute TMAs. We speculate that a reduction in plasma DNase1 activity may lead to the accumulation of pro-thrombotic NETs in patients and thus trigger excessive microvascular thrombosis as found in patients with acute TMA. NETs promote thrombosis by stimulating platelet adhesion and aggregation as well as fibrin formation in vitro and in animal models [2, 34]. Furthermore, NETs provide scaffolding for blood clots similar to fibrin strands and/or ultra-large von Willebrand factor (ULVWF) [1]. Consequently, efficient thrombolysis requires the digestion of NETs in addition to fibrin and ULVWF. Cleavage of fibrin and ULVWF is mediated by plasmin and ADAMTS13 in plasma, respectively. It is conceivable that plasma DNase1 may mediate the degradation of NETs in thrombi and thus exhibit antithrombotic effects similar to plasmin and ADAMTS13.

The role of plasma DNase1 in thrombotic diseases, however, is poorly understood. DNase1 in the circulation is increased in patients with myocardial infarction [35], but the pathophysiological significance of this finding is unknown. Antithrombotic effects of DNase1 have been described in experiments using rhDNase1 or DNase1 purified from bovine pancreas. *In vitro*, DNase1 disassembles the NET-DNA scaffold and prevents activation of platelets and clotting factors. *In vivo*, rhDNase1 inhibits experimental thrombosis [4, 14] and myocardial infarction [36] when infused prophylactically into mice.

The role of plasma DNase1 in experimental thrombosis has not been addressed. The lack of research on plasma DNase1 in thrombosis is mainly due to missing animal models and the unknown origin of plasma DNase1. DNase1 knock-out mice have been generated [37]. However, the use of these animals in experimental studies is limited due to an additional deletion-mutation in the last exon of the *Trap1/Hsp75* gene (tumor necrosis factor receptor-associated protein 1 /heat shock protein 75), which partially overlaps with the *Dnase1* gene [38].

DNase1 activity is detected in a variety of tissues and body fluids [27, 39], but the source of plasma DNase1 is, as of yet, not clearly identified in humans. DNase1 in the circulation of

humans was believed to originate from the pancreas [33]. However, DNase1 is produced by the exocrine part of the pancreas [40], which most likely excludes a direct secretion into circulation. Interestingly, hepatocytes are a major source of plasma DNase1 in mice [41]. Future studies may identify the origin of plasma DNase1 in humans and generate animal models, which specifically lack DNase1 in circulation, to study the role of plasma DNase1 in thrombosis.

The pathophysiological consequences of DNase1 deficiency are best understood in the context of autoimmunity. DNase1 is the candidate endonuclease for the breakdown of DNA from apoptotic and necrotic cells [42]. Defects in the removal of cellular debris, including DNase1 deficiencies, have been shown to induce anti-nuclear immunity, contributing to the pathogenesis of many autoimmune diseases, including systemic lupus erythematosus (SLE), thyroiditis, and inflammatory bowel disease (IBD) [43-45]. Recently, NETs were identified as a potential source of autoantigens in autoimmunity. SLE patients develop antibodies against major components of NETs such as chromatin and neutrophil proteins [46, 47]. Conclusively, an effective clearance mechanism of NETs may be essential to prohibit autoimmunity. Indeed, Hakkim et al. showed that serum from SLE patients degrades NETs less efficiently than control serum [22]. Interestingly, supplementation of SLE serum with DNase1 did not restore NET-degradation in a subset of patients because of DNase1 inhibitors and/or anti-NET antibodies in SLE serum, which block DNase1 activity and protect NETs from DNase1 digestion, respectively [22].

We observed efficient NET-degradation by TMA patient plasma supplemented with rhDNase1. These findings indicate that impaired NET-degradation in TMA patients is not due to DNase1 inhibitors or anti-NET antibodies as in some SLE patients, but a consequence of reduced levels of plasma DNase1. The lower levels of plasma DNase1 in TMA patients might be due to the consumption and/or degradation of plasma DNase1 during excessive microvascular thrombosis in TMA patients. Hemolysis is present in all forms of TMA and certain drugs are probably given to patients with neoplasia-associated TMA. Whether hemolysis or drugs can contribute to DNase1-deficiency is unknown and should be addressed in future studies. Alternatively, TMA patients may harbor an altered DNase1 gene because DNase1-reduction in SLE has been associated with genetic mutations, which reduce the half-life of DNase1 [45]. Further studies with larger patient cohorts are required to determine the molecular cause(s) of reduced DNase1 levels in TMA patients.

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Restoration of plasma DNase1 activity may become a new target for therapies of TMA patients. DNase1 infusion could compensate the DNase1 reduction and potentially ameliorate the disease. Clinical investigations have tested the therapeutic effect of DNase1. RhDNase1 (Dornase alpha, Roche) is used as aerosol by patients with cystic fibrosis for reducing sputum viscosity [48] presumably due to DNase1 mediated degradation of NETs or bacterial DNA in the sputum of these patients [49]. DNase1 has been tested in SLE patients as well. A clinical trial determined the safety and pharmacokinetics of infused rhDNase1 in patients with lupus nephritis [50]. RhDNase1 was well tolerated, but serum markers of disease activity were unchanged during the study period. Given the increasing evidence for the pro-thrombotic activity of NETs, future studies could potentially show that DNase1 infusion is clinically effective for the treatment in patients with acute TMAs or other thrombotic diseases.

Addendum

M. Jiménez-Alcázar designed and performed experiments and wrote the manuscript; R. Panda and E. C. Köhler performed experiments; M. Napirei designed experiments, provided reagents and wrote the manuscript. H. G. Mannherz performed experiments and provided reagents. S. Peine provided reagents. J. A. Kremer-Hovinga and B. Lämmle provided patient plasma samples. T. Renné provided funding, designed experiments and wrote the manuscript. B. Lämmle designed experiments and wrote the manuscript. T. A. Fuchs designed experiments, wrote the manuscript and overviewed the study.

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Disclosure of Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

Additional Supporting information is available in the online version of this article:

Table S1. Summary of demographic and diagnostic data from TMA patients and healthy controls. The cohort included plasma from 10 healthy controls (Ctrl), 3 patients with typical HUS, 7 patients with tumor-associated TMA, 9 patients with TMA of unknown etiology (NOS, not otherwise specified) and 8 patients with acute acquired TTP.. The table displays sample number, diagnosis, gender (Male/Female), age at first plasma sampling (years), ADAMTS13 activity (%), ADAMTS13 inhibitor (Bethesda units/ml), DNA (ng/ml) and DNase1 (mU/ml).

Fig. S1. EDTA inhibits degradation of NETs by plasma.

Immunofluorescence of activated neutrophils (PMA, 6h) exposed to plasma (5%) from healthy donors in the absence (Vehicle) or presence of EDTA (5mM). Samples were stained with the DNA intercalating dye SytoxGreen (green, SYTOX) or with antibodies against DNA (red, anti-DNA Ab). NETs and nuclei are visible in EDTA-treated samples. In the absence of EDTA, NETs are digested by plasma and only nuclei are visible. Scale bar = 100 μ m.

Fig. S2. Immunodetection of DNase1 in human plasma.

(A) Quantification of DNase1 by a commercial ELISA kit. We detected concentrations of purified DNase1 (provided with the kit) ranging from 0.25 to 4.5 ng/ml. No signal was obtained in samples containing 20% human plasma or serum. (B) Representative result of a Western blot employing a commercially available polyclonal antibody against DNase1. One U/ml of rhDNase1 was used as positive control. No specific band for DNase1 was detected in the plasma samples under reducing (+DTT) or non-reducing conditions (-DTT).

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Figure legends

Fig 1. Plasma from healthy donors degrades NETs efficiently. (A, B) Fluorescent DNA staining. Neutrophils were activated to release NETs before incubation with (A) buffer or (B) 5% plasma from a healthy control donor for 6 hours. NETs are shown as abundant DNA fibers. Nuclei appear as a dotted DNA staining pattern (arrows). Bars: 100 μ m. (C) Quantification of nuclei per field of view (FOV; n = 5; mean \pm SD; n.s. not significant, Statistical analysis by Mann-Whitney test). (D) Quantification of DNA fluorescence of nuclei and NETs incubated with indicated concentrations of plasma from healthy control donors for 6 hours. Shaded area indicates fluorescence emitted from nuclei (n = 14, *: p < 0.01; #, p < 0.001; §, p < 0.0001 vs. buffer, Statistical analysis by Dunn's multiple comparisons test).

Fig. 2. NET-degradation by plasma from TMA patients is impaired. (A) Fluorescent DNA staining. Neutrophils were activated to release NETs before incubation with 5% plasma from TMA patients (n = 27; HUS, n = 3; TTP, n = 8; Tumor-associated TMA, n

= 7; not otherwise specified, NOS, n = 9) or healthy control plasma (n = 10). Bar: 200 μ m. All control samples and 6/27 patient plasma samples (asterisks) degraded NETs completely. (B) Quantification of DNA fluorescence of nuclei and NETs incubated with buffer, plasma from healthy controls and plasma from TMA patients. (#, p < 0.01 vs. TMA; §: p < 0.0001 vs. buffer, Statistical analysis by Dunn's multiple comparisons test).

Fig. 3. DNase activity is decreased in TMA patient plasma. (A) Comparison of DNase activity in plasma from 10 healthy control donors and 27 patients with acute TMA (Statistical analysis by Mann-Whitney test). DNase activity is calculated in mU/ml using rhDNase1 as a standard. (B) Correlation of NET-degradation activity shown in Figure 2B and SRED DNase activity shown in Figure 3A. Strongly impaired NET-degradation indicated by DNA-fluorescence of 50% or higher, was observed in samples containing 0.12 mU/ml (dotted line) or less of DNase activity.

Fig. 4. NET-degradation requires plasma DNase1 activity. (A) Fluorescence DNA staining of nuclei and NETs incubated with plasma from five healthy controls (Ctrl Pls 1 – 5) or buffer (B 1 – 5). Plasma was supplemented with vehicle (V) or actin:G1 (A, 5 μ M). (B) Quantification of DNA fluorescence shown in panel A. Inhibition of DNase1 by actin:G1 (Actin, 5 μ M) prevents NET-degradation by plasma from healthy control donors (Statistical analysis by Paired t-test).

Fig. 5. Levels of active DNase1 are decreased in TMA patient plasma. (A) Representative examples of plasma from a healthy donor and two TMA plasma samples analyzed by the SRED and DPZ method. No DNase activity was detected by the SRED and DPZ assays in plasma of patient 1. The healthy control and the patient 2 showed plasma DNase activity in the SRED and DPZ analysis. (B) Quantification of plasma DNase activity in healthy control donors (n = 10) and patients with acute TMA (n = 27) by the DPZ method (Statistical analysis by Mann-Whitney test). (C) Detection of DNase1 inhibitors in plasma by comparing DNase activities from SRED and DPZ analysis. Pooled plasma from 5 healthy control donors was supplemented with vehicle or 5 μ M actin:G1. DNase1 inhibition was detected in samples containing actin:G1 by the SRED assay but not in the DPZ analysis. A: Albumin, D: DNase1. (D) Quantification of SRED and DPZ analysis of

plasma supplemented with different concentrations of actin:G1. (E) Correlation of plasma DNase activity in TMA patient plasma quantified by DPZ and SRED (Statistical analysis by Spearman correlation, r : Spearman's rank correlation coefficient).

Fig. 6. RhDNase1 restores NET-degradation activity of TMA patient plasma. (A) Quantification of plasma DNase activity by the SRED assay. Plasma from 5 TMA patients (TMA Plasma 1 – 5) was supplemented with vehicle (V) or rhDNase1 (D; 500 μ U/ml). Plasma from 5 healthy donors was analyzed as control (Ctrl 1 – 5). (B) Quantification of DNase activities shown in panel A (Statistical analysis by Paired t-test). Supplementation with rhDNase1 restores the DNase activity of TMA patient plasma to levels of healthy controls. (C) Fluorescent DNA staining of nuclei and NETs from activated neutrophils, which were incubated with plasma from 5 TMA patients (1 – 5) or buffer (B, 1 - 5). TMA patient plasma was supplemented with vehicle (V), rhDNase1 (500 μ U/ml, D), or mixed with plasma from healthy controls (1 vol/1 vol, P). Bar: 200 μ m. (D) Quantification of DNA fluorescence shown in panel C (Statistical analysis by Paired t-test). Supplementation of TMA patient plasma with rhDNase1 or plasma from healthy donors restored the NET-degradation activity.

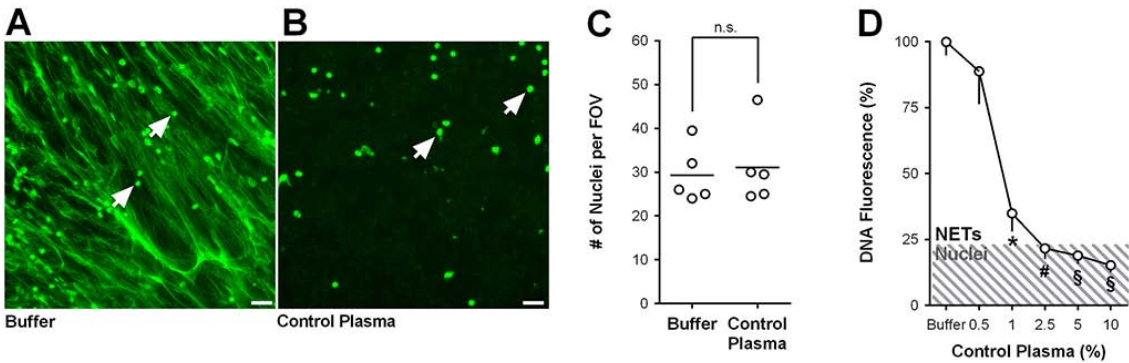


Figure 1

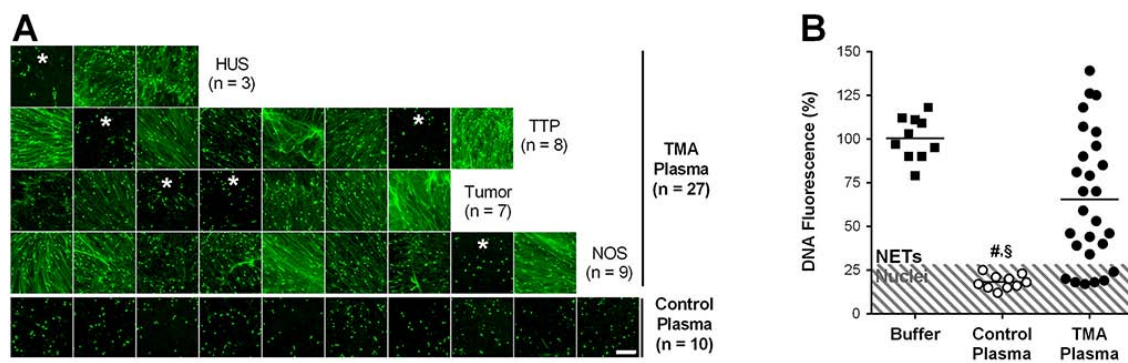


Figure 2

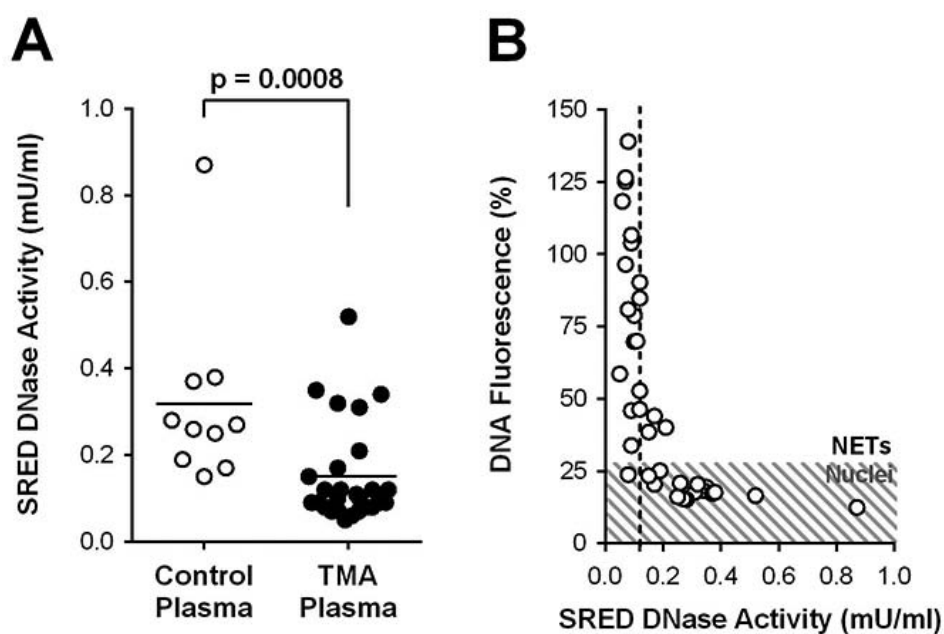


Figure 3

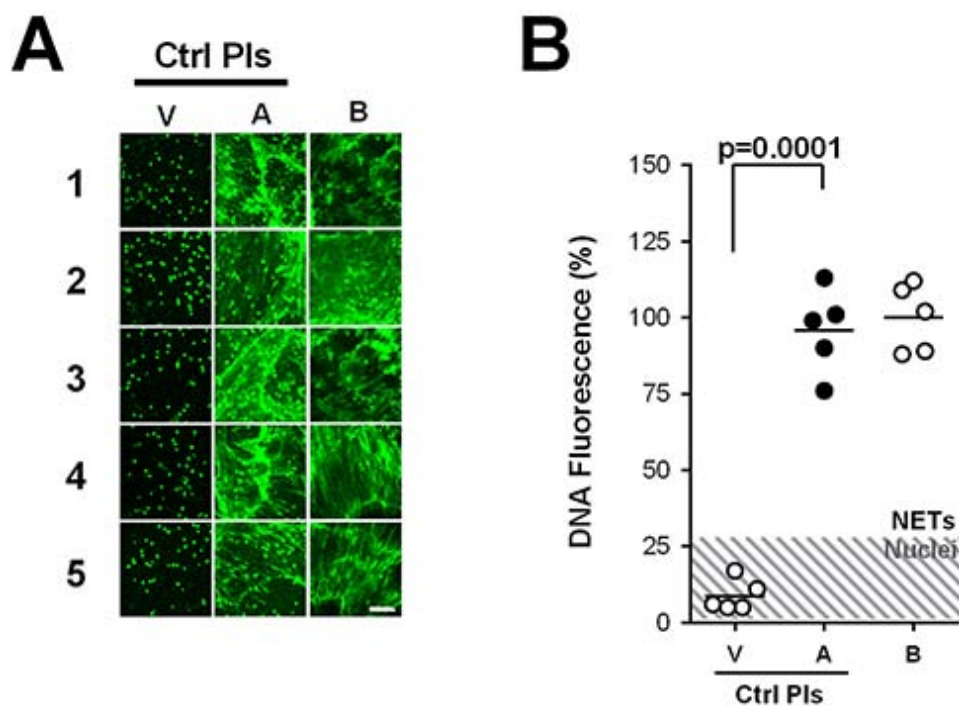


Figure 4

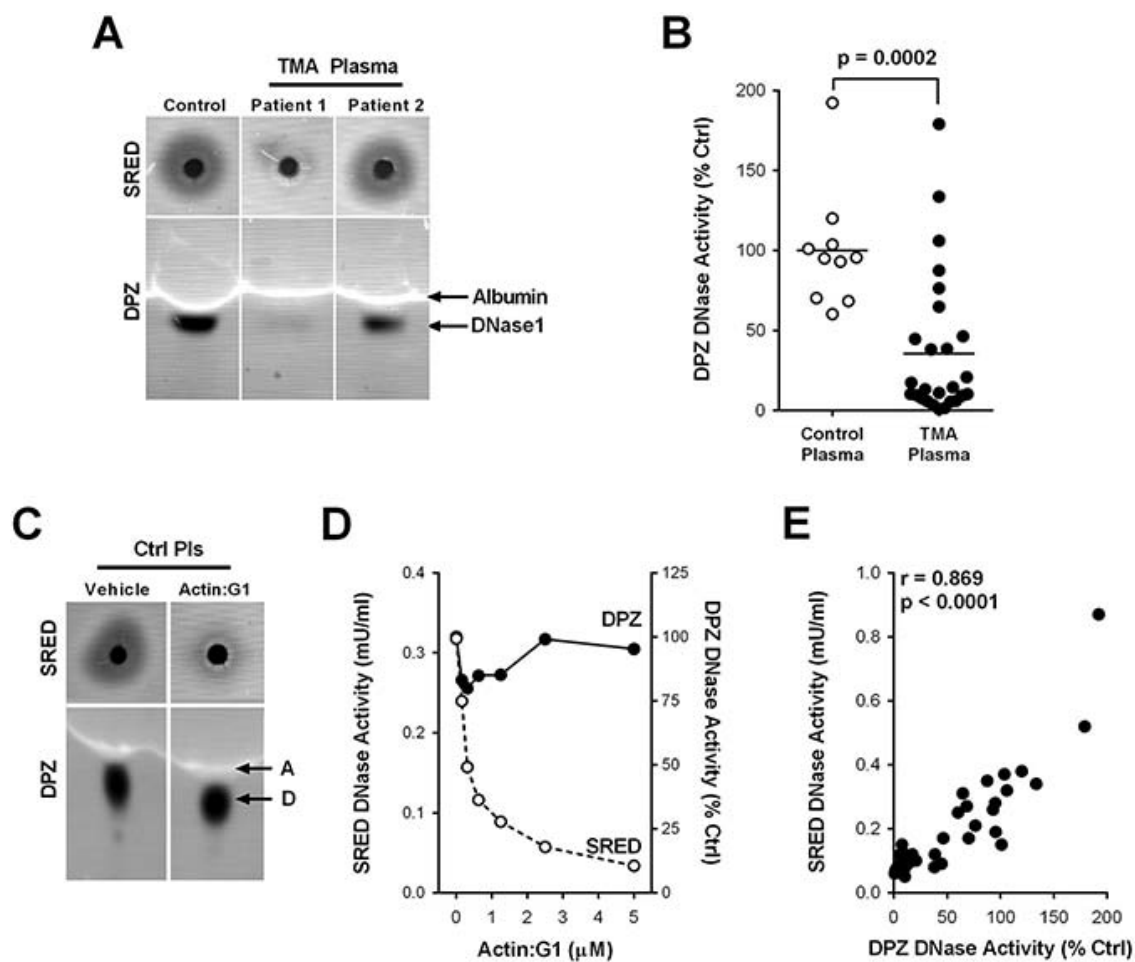


Figure 5

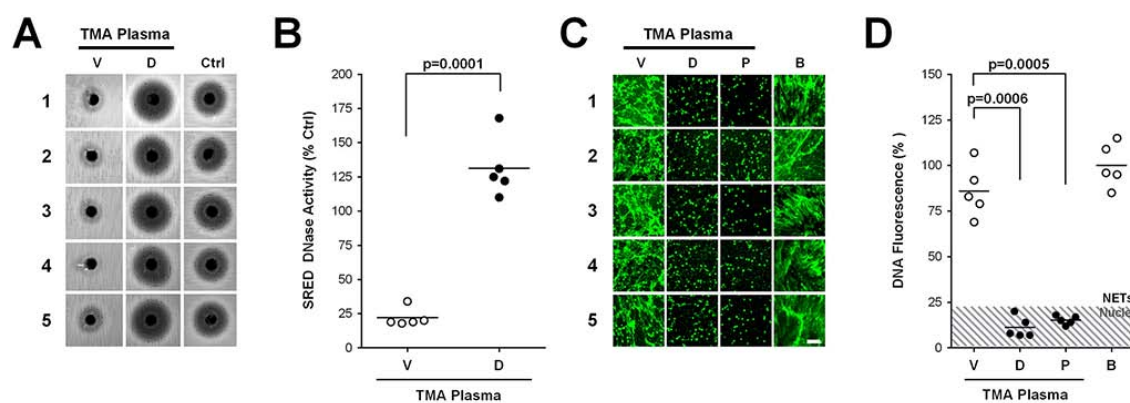


Figure 6